

Soluble Markers of the Toll-Like Receptor 4 Pathway Differentiate between Active and Latent Tuberculosis and Are Associated with Treatment Responses

Siri L. Feruglio¹, Marius Trøseid², Jan Kristian Damås^{3,4}, Dag Kvale^{1,2}, Anne Ma Dyrhol-Riise^{1,2,5*}

1 Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway, **2** Department of Infectious Diseases, Oslo University Hospital, Oslo, Norway, **3** Department of Infectious Diseases, St. Olavs Hospital, Trondheim, Norway, **4** Institute of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway, **5** Department of Clinical Science, University of Bergen, Bergen, Norway

Abstract

Background: Biomarkers to differentiate between active tuberculosis (TB) and latent TB infection (LTBI) and to monitor treatment responses are requested to complement TB diagnostics and control, particularly in patients with multi-drug resistant TB. We have studied soluble markers of the Toll-like-receptor 4 (TLR-4) pathway in various stages of TB disease and during anti-TB treatment.

Methods: Plasma samples from patients with culture confirmed drug-sensitive TB ($n = 19$) were collected before and after 2, 8 and 24 weeks of efficient anti-TB treatment and in a LTBI group ($n = 6$). Soluble (s) CD14 and myeloid differentiation-2 (MD-2) were analyzed by the Enzyme-linked immunosorbent assay (ELISA). Lipopolysaccharide (LPS) was analyzed by the Limulus Amebocyte Lysate colorimetric assay. Nonparametric statistics were applied.

Results: Plasma levels of sCD14 ($p < 0.001$), MD-2 ($p = 0.036$) and LPS ($p = 0.069$) were elevated at baseline in patients with untreated active TB compared to the LTBI group. MD-2 concentrations decreased after 2 weeks of treatment ($p = 0.011$), while LPS levels decreased after 8 weeks ($p = 0.005$). In contrast, sCD14 levels increased after 2 weeks ($p = 0.047$) with a subsequent modest decrease throughout the treatment period. There was no significant difference in concentrations of any of these markers between patients with pulmonary and extrapulmonary TB or between patients with or without symptoms.

Conclusion: Our data suggest that plasma levels of LPS, MD-2 and sCD14 can discriminate between active TB and LTBI. A decline in LPS and MD-2 concentrations was associated with response to anti-TB treatment. The clinical potential of these soluble TLR-4 pathway proteins needs to be further explored.

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* E-mail: a.m.d.riise@medisin.uio.no

Introduction

Tuberculosis (TB) is a global challenge and there were an estimated 8.7 million incident cases in 2011, 13% were co-infected with human immunodeficiency virus (HIV) and 20% of previously treated cases had multi-drug resistant (MDR)-TB [1]. Both TB culture, which is the diagnostic gold standard, and the *Xpert MTB/RIF* assay often fail in cases of extrapulmonary TB, in HIV-infection and in children [2] and TB diagnosis is often made on clinical criteria. Thus, leading stakeholders have called for intensified basic scientific research to explore TB immunopathogenesis and screen for diagnostic and prognostic biomarker candidates that could complement diagnosis [3–5]. Predictors of treatment efficacy are particularly relevant in MDR-TB with less efficient therapeutic regimens of longer duration.

A number of TB antigens have been tested as potential biomarkers [6,7]. Available serology tests are unreliable even though they are in extensive use in some TB high-endemic

countries [8,9]. Several meta-analyses show that the cellular interferon- gamma- release- assays (IGRAs) are at least as sensitive and more specific than the tuberculin skin test (TST) [10–13]. Still, they do not discriminate between latent TB infection (LTBI) and active TB and cannot monitor therapy.

Mycobacterium tuberculosis (*Mtb*) possesses a unique cell envelope with an outer lipid layer of mycolic acids and intercalated additional complex lipids, which have been the targets for immunogenic, diagnostic and drug research [14]. *Mtb* synthesizes two unique families of cytoplasmic polymethylated polysaccharides, the methylglucose lipopolysaccharides (MGLPs) and the methylmannose polysaccharides (MMPs) [15–17]. Cell wall-associated lipoglycans such as lipoarabinomannan (LAM) inhibit the expression of pro-inflammatory cytokines by activated macrophages and has been identified as a major virulence factor of *Mtb* [18]. Whereas the point-of-care LAM urine antigen test could aid in the diagnosis of advanced HIV-associated TB [19],

Table 1. Demographic and clinical characteristics of study participants.

		Active TB	Latent TB
		N = 19	N = 6
Median age (years)¹		32 (26–41)	40 (26–54)
Sex	Female	9	4
	Male	10	2
Country of origin	Africa	9	2
	Asia	6	2
	Europe	4 ²	2 ³
Localisation of disease	Pulmonary	10	
	Extrapulmonary	9	
Symptomatic TB⁴		9	

¹Interquartile range in brackets.²Including two native Norwegians.³All native Norwegians.⁴≥2 of the following symptoms: fever (temperature>38°C), weight loss, wasting, cough and night-sweat.

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the overall low sensitivity in HIV negative TB patients may still limit this test's utility [20].

Toll-like receptors (TLR), essential membrane receptors that recognize pathogen-associated molecules, interact with specific *Mtb* structures triggering signaling pathways [21]. Various types of LPS induce synthesis of proinflammatory cytokines through binding to the TLR-4/myeloid differentiation-2 (MD-2) complex [22,23]. Several soluble proteins regulate the signaling of LPS through the TLR-4 pathway. CD14, a glycoprotein expressed on the surface of macrophages, monocytes and phagocytes seems to be essential in the host response against microbial infection [24,25]. Soluble (s) CD14 serves as a receptor for the complex of LPS and LPS binding protein (LBP) and plays a central role by transferring LPS to MD-2, thus activating immune responses to bacterial endotoxins [22,26]. Elevated levels of sCD14 are associated with various systemic inflammatory diseases including TB [27], and increased levels of MD-2 have been detected in plasma from patients with severe sepsis as well as in HIV infection [28,29]. It has been shown that during anti-TB treatment the levels of sCD14 slowly decline [27] and the levels of LBP are lower in treated compared to untreated TB patients [30]. To our knowledge, however, there are no longitudinal studies of circulating levels of MD-2, sCD14 and LPS in TB patients or of the possible effects of anti-TB treatment upon these potential biomarkers. Our pilot study indicates that plasma levels of LPS and MD-2 can discriminate between various stages of TB disease and possibly predict responses to therapy.

Materials and Methods

Study Participants

Nineteen patients with active culture-confirmed TB, ten with pulmonary and nine with extrapulmonary TB, were recruited from the Department of Infectious Diseases, Oslo University Hospital, Norway. Demographical and clinical data were as described in Table 1. Two patients were native Norwegians (age 81 and 92) and the remaining were immigrants, predominately from Africa (n=9) and Asia (n=6). Patients were classified as symptomatic when they presented with two or more of the following symptoms: fever (temperature>38°C), weight loss,

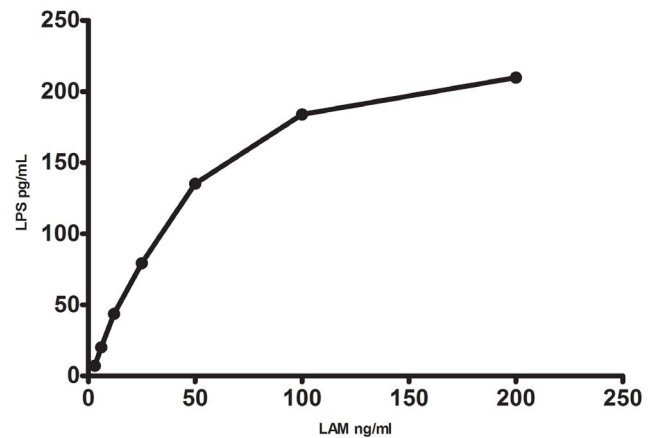


Figure 1. Lipoarabinomannan (LAM) detected by the Lipopolysaccharide (LPS) assay. Relation between Lipoarabinomannan (LAM) standard concentrations from the Enzyme-linked immunosorbent assay (ELISA) kit and Lipopolysaccharide (LPS) concentrations analysed by the Limulus Amebocyte Lysate colorimetric assay (LAL). doi:10.1371/journal.pone.0069896.g001

wasting, cough and night-sweat. Nine had symptomatic TB while the others were diagnosed in routine screening or investigations for other medical conditions. The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), thoracic X ray, acid-fast staining and culture of sputum results were registered upon inclusion. All *Mtb* strains were drug-sensitive. Patients were treated with a standard combination of four drugs (isoniazid, rifampicin, ethambutol and pyrazinamide) for 2 months, followed by isoniazid and rifampicin for 4 months. Sputum samples were assessed after 2 and 6 months of treatment and patients were also evaluated after one year according to clinical practice. All patients responded to treatment, pulmonary TB patients with sputum conversion and culture clearing whereas extrapulmonary TB patients demonstrated clinical improvement and normalization of inflammation parameters (CRP, ESR). No relapse was observed during the year of follow-up. Six subjects with LTBI were recruited from Haukeland University Hospital, Bergen. Two were native Norwegians and four immigrants. All LTBI tested positive with the QuantiFERON-TB® test (Cellestis/Qiagen). Thoracic X-ray, sputum smears and clinical examinations were performed and there were no signs of active TB in any of the persons. Both active TB and LTBI patients were HIV negative.

Blood samples were obtained by venipuncture prior to start of treatment (baseline), after 2, 8 and 24 weeks in the active TB group and at the time of diagnosis in the LTBI group. Plasma samples were separated and snap-frozen within 30 minutes and stored at minus 80°C until analysis.

The study was approved by the respective Regional Boards for Medical and Health Research Ethics in Norway (in Health region South-East and West). Written informed consent was obtained from all participants.

Lipopolysaccharide Assay

We observed that the Limulus Amebocyte Lysate colorimetric assay (LAL) (Lonza, Walkersville, MD, USA) could detect TB-specific LAM from the General LAM Enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Eiaab Science, Whuan, China) in a dose dependent manner, indicating that the LAL assay could detect mycobacterial LPS (Fig. 1). We then analyzed LAM in plasma samples from the active TB group in 8- and 24-fold dilutions according to the manufacturer's instructions. However,

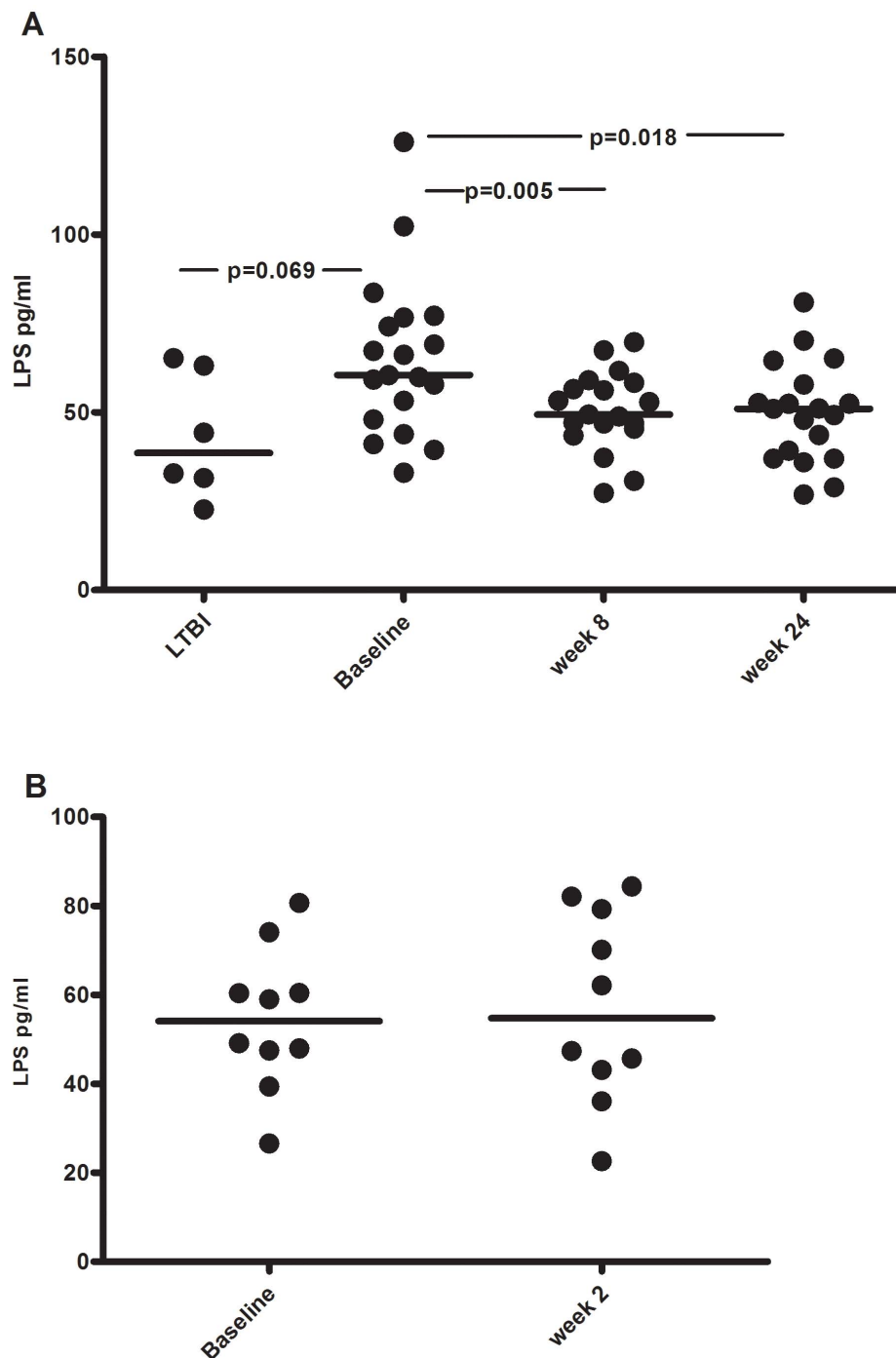


Figure 2. Lipopolysaccharide levels at various stages of TB infection and during therapy. Lipopolysaccharide (LPS) concentrations (pg/ml) in plasma from A) patients with LTBI ($n=6$) and active TB ($n=19$) at baseline and after 8 and 24 weeks of anti-TB therapy and B) active TB ($n=10$) at baseline and after 2 weeks of anti-TB therapy. Horizontal lines represent the median values. Significant p values ($<.05$) between groups and time-points are indicated.

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about 40% of the tested samples were below twice the background optic density value and thereby not quantifiable (data not shown).

LPS concentrations in plasma were then analyzed in duplicates by the LAL colorimetric assay according to the manufacturer's instructions with the following modifications: plasma samples were diluted 10-fold to avoid interference with background color and preheated to 68°C for 12 minutes prior to analyses to dissolve

immune complexes as previously described [29]. Test samples were mixed with the LAL supplied in the test kit and incubated at 37°C for 10 minutes. A substrate solution was then mixed with the LAL sample and incubated at 37°C for an additional 6 minutes and the reaction was stopped. A conversion to yellow developed if LPS was present. The absorbance of the sample was determined

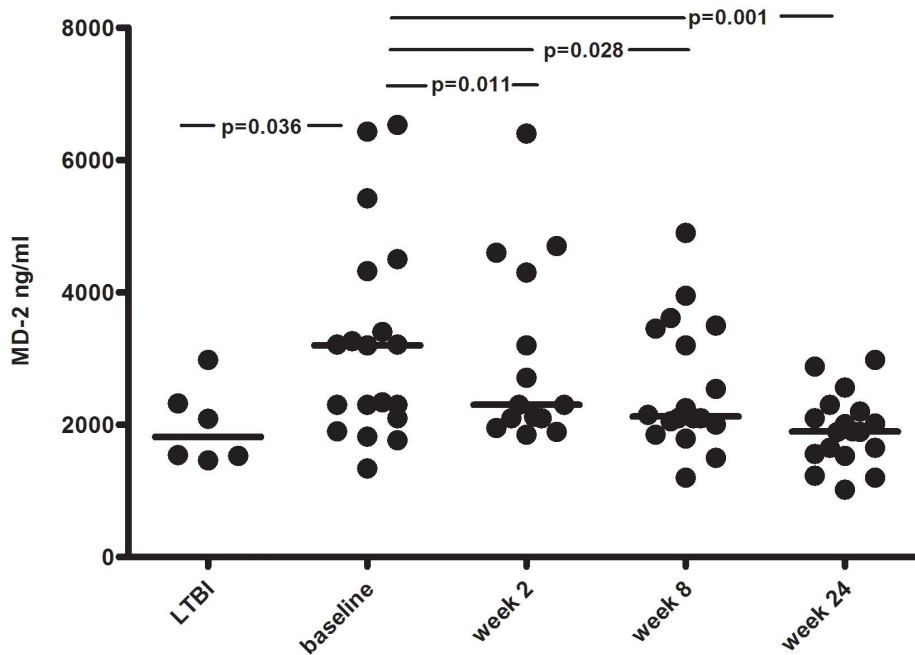


Figure 3. Myeloid differentiation-2 levels at various stages of TB infection and during therapy. Myeloid differentiation-2 (MD-2) concentrations (ng/ml) in plasma from patients with LTBI (n=6) and active TB (n=19) at baseline and after 2, 8 and 24 weeks of anti-TB therapy. Horizontal lines represent the median values. Significant p values (<.05) between groups and time-points are indicated. doi:10.1371/journal.pone.0069896.g003

by spectrophotometer and concentration calculated from a standard curve.

Myeloid Differentiation-2 ELISA

MD-2 was analyzed by an in-house ELISA as previously described [31]. Briefly, MD-2 was captured on immunoplates coated with a TLR-4-Fc fusion protein and detected with digoxigenin labeled MD-2 mAb 5D7 or digoxigenin labeled IIC1 and anti-digoxigenin-HRP (Roche). The MD-2 standard was purchased from R&D Systems.

Soluble CD14 ELISA

sCD14 was analyzed by a sandwich ELISA coated with CD14-specific monoclonal antibodies according to the manufacturer's instructions (R&D, Minneapolis, MN, USA). Samples were diluted 300-fold and incubated in duplicates for three hours at room temperature. After a total of four washing steps, the wells were incubated with sCD14 conjugate solution for one hour. The cells were washed, substrate solution added, incubated for 30 minutes and stopped. A conversion to blue developed if sCD14 was present, the absorbance of the sample was determined by spectrophotometer and concentrations were calculated from a standard curve.

Inflammation Markers

CRP was estimated by Immuno Turbidometric assay (Cobas 8000, Roche Diagnostics, USA) and ESR according to Westergren Method (StaRRsed Compact Automatic Analyzer, RRmechatronic, Hoom, the Netherlands).

Statistical Analysis

Statistical analyses were performed by Statistica v 7.0 (Statsoft, Tulsa, OK, USA). Nonparametrical statistical methods were applied for group-wise comparison. Mann-Whitney U test and

two-tailed Wilcoxon matched pairs test for dependent variables. Correlation analyses were performed using the Spearman method. A significance level of 0.05 was used. All values are presented as median and interquartile range (IQR).

Results

Elevated Plasma Lipopolysaccharide Levels Decline during TB Treatment

Plasma levels of LPS at baseline were higher, although not significant, in the active TB group compared to the LTBI group (60 pg/mL [44–77] vs. 39 pg/mL [31–63], $p = 0.069$) (Fig. 2A). In the active TB group LPS levels decreased significantly during treatment (49 pg/mL [43–57], $p = 0.005$ at week 8). The decline in LPS level was maintained at week 24 (48 pg/mL [37–52], $p = 0.018$). No significant decline was observed from baseline to week 2, measured in ten of the patients (Fig. 2B). LPS concentrations did not differ significantly between patients with pulmonary and extrapulmonary TB (60 pg/mL [44–69] vs. 59 pg/mL [48–77]).

Elevated Plasma Myeloid Differentiation-2 Levels Rapidly Decline during TB Treatment

Plasma levels of MD-2 at baseline were higher in the active TB group compared to the LTBI group (3200 ng/mL [2100–4320] vs. 1815 ng/mL [1530–2320], $p = 0.036$) (Fig. 3). In active TB patients there was a rapid decline in the level of MD-2 compared to baseline, significant already after 2 weeks of treatment (2300 ng/mL [2100–4300], $p = 0.011$), and still significant at week 8 (2125 ng/mL [2000–3450], $p = 0.028$) and week 24 (1895 ng/mL [1560–2200], $p = 0.001$). MD-2 concentrations did not differ significantly between patients with pulmonary and extrapulmonary TB (2655 ng/mL [1820–5420] vs. 3200 ng/mL [2300–3260]).

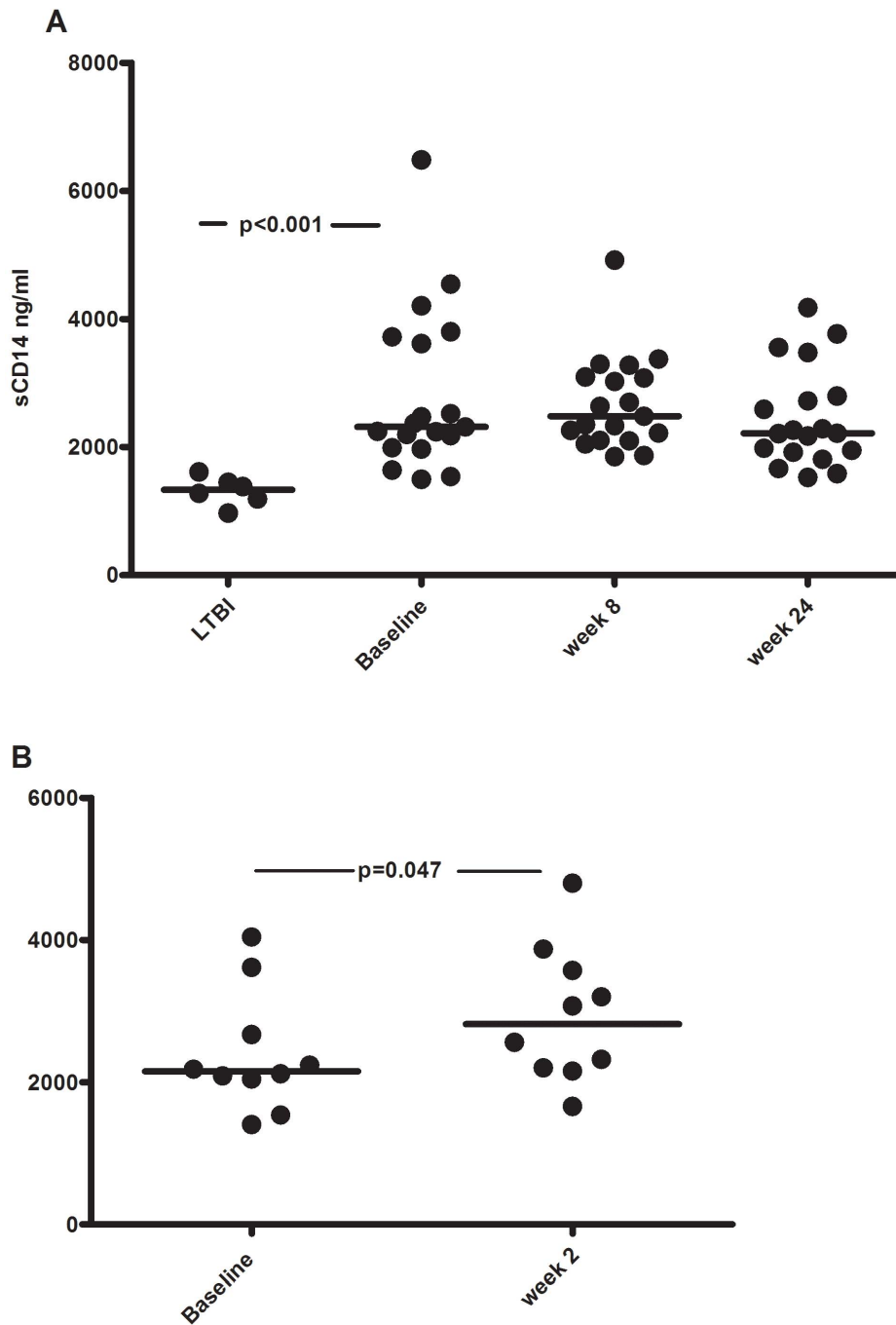


Figure 4. sCD14 levels at various stages of TB infection and during therapy. Soluble CD14 concentrations (ng/ml) in plasma from A) patients with LTBI (n = 6) and active TB (n = 19) at baseline and after 8 and 24 weeks of anti-TB therapy and B) active TB (n = 10) at baseline and after 2 weeks of anti-TB therapy. Horizontal lines represent the median values. Significant p values (<0.05) between groups and time-points are indicated. doi:10.1371/journal.pone.0069896.g004

Elevated Plasma Levels of sCD14 during TB Treatment

Plasma levels of sCD14 at baseline were higher in the active TB group compared to the LTBI group (2320 ng/ml [1990–3720] vs. 1335 ng/ml [1190–1450], $p < 0.001$) (Fig. 4A). After 2 weeks of therapy there was a significant increase in sCD14 in the active TB group (2820 ng/ml [2190–3650], $p = 0.047$) (Fig. 4B). A slightly decrease in median sCD14 was observed after 24 weeks of therapy (2220 ng/ml [1920–2800]), although not significant ($p = 0.064$) (Fig. 4A). sCD14 levels did not differ between patients with

pulmonary and extrapulmonary TB (2450 ng/ml [2200–3720] vs. 2240 ng/mL [1640–2475]).

Correlations between sCD14, LPS, MD-2 and Inflammation Markers

Nine active TB patients were categorized as symptomatic at baseline. The inflammation markers CRP and ESR were significantly higher in the symptomatic compared to the asymptomatic TB group (77 mg/L [36–95] vs. 24 mg/L [3–18],

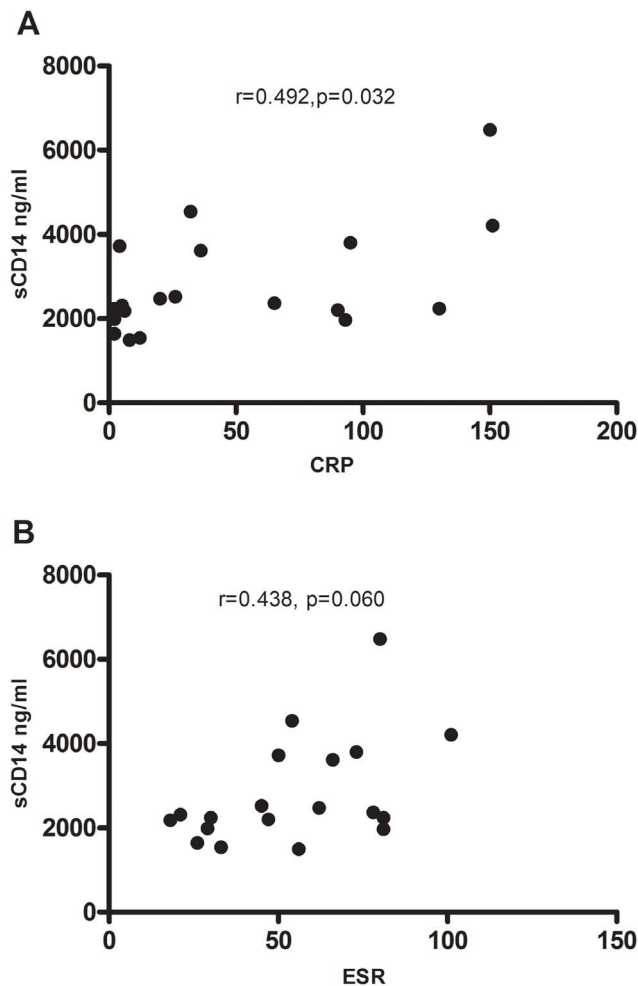


Figure 5. Correlations between sCD14 and inflammation markers. Correlations between inflammation markers in the active TB group (n=19) between A) CRP and sCD14 ($r=0.492$, $p=0.032$), B) ESR and sCD14 ($r=0.438$, $p=0.060$). doi:10.1371/journal.pone.0069896.g005

$p=0.017$ and 67 mm [50–80] vs. 43 mm [27–56], $p=0.028$, respectively), but CRP and ESR could not discriminate between pulmonary and extrapulmonary TB. At baseline there were significant correlations between CRP and sCD14 ($r=0.492$, $p=0.032$) while the correlation between ESR and sCD14 was modest ($r=0.438$, $p=0.060$) (Fig. 5). LPS or MD-2 did not correlate to either CRP or ESR. There were no significant differences in levels of LPS, sCD14 and MD-2 between patients with high or low symptom score or between patients with pulmonary and extrapulmonary TB (data not shown).

Discussion

Mycobacterial cell wall components are immunogenic and thus potential biomarkers for TB [14]. Proteins involved in the regulation of innate immunity through the TLR-4 pathway could also serve as surrogates for TB disease [21]. We have analyzed plasma concentrations of LPS, sCD14 and MD-2 in patients with various clinical manifestations and stages of TB infection and during anti-TB treatment. We have shown that plasma levels of MD-2, sCD14 and possibly LPS are higher in patients with active TB than in LTBI. Most important, we found that the decline in

LPS and MD-2 concentrations was associated with response to anti-TB treatment. sCD14 transfers LPS to MD-2, thus activating immune responses to bacterial endotoxins [22,26]. Immunoregulatory cytokines such as IL-10 downregulate protective immune responses in chronic active TB infection [32] and seems to be a driving force for increased release of MD-2 and CD14 during systemic inflammation [31]. Thus, the decline in these markers, for MD-2 measured already after 2 weeks, could reflect an early down-regulation of immune activation during effective anti-TB therapy. The decline in LPS levels supports an Indian study where they isolated an immunodominant LPS antigen from *Mtb* H37Rv and compared LPS with three commercially available *Mtb*-specific antigens for detection of TB [33]. They found that LPS sensitivity was 84% and specificity 97%, suggesting that the LPS antigen could be used in sero-diagnosis of TB. Further, a recent study on circulating biomarkers demonstrated that LPS was elevated in plasma from children with pulmonary TB, arguing that LPS might serve as a marker of innate immune activation [34]. Previous studies have also shown that proteins involved in the innate immune activation are elevated in other infectious diseases as parasitic infections [35], HIV infection [36] and hepatitis [37]. Increased circulating LPS could result from chronic immune activation caused by microbial translocation as has been demonstrated in HIV infection [36,38]. We think, however, that in our patients this is less likely as none had gastrointestinal or disseminated TB. In contrast to that found for MD-2 we did not record any changes in LPS levels after 2 weeks of treatment. Wood *et al* showed a decline in urine LAM concentrations in South-African patients treated for active TB between 2 and 8 weeks of treatment [39], and given the apparently higher sensitivity of our LAL-assay, further investigations of the time kinetics of LPS during anti-TB should be performed.

We found higher levels of sCD14 in plasma from patients with active TB compared to those with LTBI, as shown in previous studies [27,30]. We observed a very modest and not statistically significant decline of sCD14 throughout the 24 week observation period. Whereas Juffermans *et al* found normalization of sCD14 levels in TB patients that had completed curative treatment [30], Lawn *et al* showed that the levels of sCD14 declined slowly and were still significantly elevated after 3 months of treatment, in support of our data [27].

Still, we detected increased plasma levels of sCD14 at week 2 of TB treatment in a smaller group of patients where plasma samples were available. Lawn *et al* also observed a similar transient increase during the first month of treatment, followed by a slow decline in serum sCD14 concentrations [27]. They argue that the initial rise in sCD14 levels might represent the host response to the increase of mycobacterial antigen load due to therapy corresponding to what has been previously shown for tumour necrosis factor alpha (TNF- α) [40]. Our data supports this conclusion, although the mechanisms need to be further documented by *in vitro* studies.

In our study sCD14 levels correlated to some extent to CRP levels, suggesting that both these markers reflect the level of inflammation [41]. In contrast, we did not find correlations between inflammation markers and LPS or MD-2 plasma levels, respectively. It has been shown that cell wall-associated lipids inhibit LPS-induced TNF- α release by a sCD14-dependent pathway, while IL-12 p40 inhibition is sCD14-independent [42]. Such mechanisms could explain the diverse associations detected for the various markers.

In our study the parameters did not discriminate between either disease localization or symptom score. One would assume significantly lower plasma levels of sCD14 in extrapulmonary TB compared to pulmonary TB due to the expected various levels

of bacillary load [30]. However, all extrapulmonary TB patients in our study had positive TB cultures and neither CRP nor ESR could discriminate between pulmonary and extrapulmonary TB. In fact, several of the extrapulmonary cases had much higher levels of inflammation markers than that found in many of the patients with pulmonary TB. This, as well as the relatively low numbers of patients in each group, could explain why we did not find any significant difference for sCD14, LPS and MD-2 between these two patient groups. Of note, the rapid decline in MD-2 in response to therapy was also seen in those patients with none or few symptoms where clinical improvement would be of less use as parameter of therapy efficacy. Thus, MD-2 could potentially serve as a complimentary biomarker when evaluating the initial response to therapy in patients with few symptoms.

The limitation of this study is the small sample size, which increases the risk of type II statistical errors. However, since type I errors are less likely, both our findings that MD-2 and sCD14 can discriminate between latent and active TB and that MD-2 and LPS are associated with treatment responses are probably valid. Further, the LAL assay might be less specific in patients with

concurrent HIV infection and increased levels of LPS due to microbial translocation [43].

In conclusion, based on our data and other reports, a multi-parameter approach including biomarkers must be considered to aid in TB diagnosis and monitor efficacy of TB treatment [5,44]. The clinical importance of these TLR-4 regulatory proteins is yet to be determined. Still, our explorative novel data indicate that these soluble markers, in particular MD-2, need to be further studied in larger cohorts of TB patients.

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Author Contributions

Conceived and designed the experiments: SF MT JD DK ADR. Performed the experiments: SF MT JD. Analyzed the data: SF DK ADR. Contributed reagents/materials/analysis tools: SF MT JD DK ADR. Wrote the paper: SF MT JD DK ADR.

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